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Published in:
Journal of the Chemical Society%2C Chemical Communications

DOI:
10.1039/c39950000255

Large Activation of Serine Proteases by Pretreatment with Crown Ethers

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Pretreatment of serine proteases by lyophilisation in the presence of crown ethers leads to large enhancements of enzyme activity in organic solvents.

The potential advantages of the use of enzymes in organic media have been well recognized in recent years. A higher stability of the enzyme and different substrate-, regio- and enantio-selectivities are some of the advantages quoted.[1] The exploitation of these advantages is, however, limited by the much lower activity (generally 2–4 orders of magnitude) of most enzymes in organic solvents than in water. Strategies to enhance the enzyme activity by protein engineering,[2] chemical modification of the enzymes,[3] immobilization,[4] lyoprotection,[5] interaction with dialkylamphiphiles,[6] and the use of 'water mimicking' compounds[7] such as formamide, glycol or DMF have been reported. We have previously demonstrated that the activity of several serine proteases[8,9] and that of tyrosinase[10] can be increased by one to two orders of magnitude by addition of crown ethers to the organic reaction medium. A macrocyclic effect was found to be responsible for the increased enzyme activity since no effect was found for an open chain analogue.[9]

Here, we report that enzyme pretreatment by lyophilisation from an aqueous crown ether solution results in enzyme samples which exhibit a dramatically increased enzyme activity in organic solvents. Table 1 shows the effect of crown ether lyophilisation of serine proteases from sodium phosphate buffer pH 7.8 with various amounts of crown ethers. The activity of the enzymes was measured in the transesterification of *N*-acetyl-L-phenylalanine ethyl ester [*N*-Ac-L-PheOEt] with propan-1-ol in cyclohexane.

Lyophilisation of α-chymotrypsin in the presence of 250 equiv. of 18-crown-6 (18-C-6) increases the activity of α-chymotrypsin 640 times, resulting in a k_{cat}/K_M value† of 770 $dm^3\ mol^{-1}\ s^{-1}$. This activity is the highest ever observed for α-chymotrypsin suspended in organic solvent, and is only 50 times lower than that of α-chymotrypsin in water.[11]

The water-soluble crown ethers 15-crown-5 (15-C-5) and 12-crown-4 (12-C-4) also activate α-chymotrypsin, although the effects are less pronounced. The smaller effects may be, at least partly, attributed to the lower amount of crown ether present in the enzyme sample, as most of the initially added 500 equiv. of these crown ethers evaporates during the lyophilisation process. Also pentaglyme, which has shown to have a negligible effect when added to suspended enzyme in organic solvent,[9] enhances the enzyme activity when it is present during lyophilisation. However, the activating effect is much lower than that of 18-C-6.

The effect of 18-C-6 lyophilisation was also investigated for some other serine proteases. The activity of subtilisin Carlsberg and trypsin is significantly increased by the crown ether lyophilisation.‡ In contrast, acetyltrypsin in which all solvent-accessible lysine ammonium and tyrosine hydroxyl groups are acetylated,[12] is not activated by the pretreatment with 18-C-6. This indicates that interactions of 18-C-6 with lysine ammonium and/or tyrosine hydroxyl groups do play a role in the activation mechanism. The lower activation of subtilisin Carlsberg compared to trypsin and α-chymotrypsin may be the consequence of the lower number of lysine residues present on the enzyme surface of this enzyme, which is 7–8, 14, and 14 for subtilisin Carlsberg, α-chymotrypsin, and trypsin, respectively.[13]

Generally, the lower activity of serine proteases in organic solvents is accompanied by a significantly lower enantioselectivity compared to aqueous solution,[14] suggesting a relation between activity and enantioselectivity. However, α-chymotrypsin lyophilised with 500 equiv. of 18-C-6 is 300 times more active than the unpretreated enzyme towards the racemic substrate *N*-acetyl-D,L-alanine 2-fluoroethyl ester,§ but the enantioselectivity of both enzyme samples is not significantly different (E = 25 and 27, respectively). This clearly shows that the enantioselectivity of α-chymotrypsin suspended in organic solvents is not directly correlated with the activity observed in these media.

Table 1 Effect of additive present during lyophilisation,[a] on the activity of serine proteases in the transesterification of *N*-acetyl-L-phenylalanine ethyl ester in cyclohexane–1 mol dm^{-3} PrOH[b]

Enzyme	Additive	Mol additive / mol enzyme	V_0(+additive)[c]	V_0(+additive) / V_0
α-Chymotrypsin		0.0	8	1.0
	18-C-6	50	330	41
		250	5100	640
		500	4700	585
		1000	620	77
	15-C-5	20[d]	460	58
	12-C-4	5[d]	180	22
	Pentaglyme	500	660	82
Subtilisin Carlsberg		0.0	15	1.0
	18-C-6	500	430	28
Trypsin		0.0	0.23	1.0
	18-C-6	500	49	216
Acetyltrypsin		0.0	0.70	1.0
	18-C-6	500	0.63	0.9

[a] Enzymes (5 mg cm^{-3}) were dissolved in 20 mmol dm^{-3} sodium phosphate buffer pH 7.8, including the appropriate amount of additive, followed by freeze-drying for 24–30 h. The enzyme samples and the organic solvent were (separately) equilibrated above a saturated LiCl solution[17] at 5 °C for at least 24 h in order to obtain a reaction medium of constant water activity. [b] Conditions: 2.5 × 10^{-3} mol dm^{-3} substrate, 1 mg cm^{-3} enzyme powder, stirring rate 350 rpm, 25 °C. [c] Initial rates (10^{-7} mol min^{-1}) per mg protein cm^{-3} were determined at least *in duplo*. Variations were less than 10%. [d] A solution with 500 equiv. was freeze dried, but due to evaporation of this additive only a fraction was left after the freeze drying was completed.

When α-chymotrypsin, lyophilised with 500 equiv. of 18-C-6, is subsequently washed with cyclohexane–1 mol dm^{-3} propan-1-ol to remove the crown ether, the resulting enzyme sample shows no enhanced activity. Moreover, when 18-C-6 is added after that the enzyme is suspended in organic solvent,[9] the enzyme activation is more than thirty times lower than by the pretreatment procedure. It was also established that 18-C-6 does not increase the activity of α-chymotrypsin in aqueous solution. Therefore, the activation by crown ether lyophilisation must originate from specific crown ether–enzyme interactions generated during the lyophilisation process and which are preserved in the organic solvent.

Time-resolved fluorescence depolarisation experiments[15] on active site labelled α-chymotrypsin have pointed out that the mobility of the probe in the active site is not significantly changed by the crown ether lyophilisation. The crown ether activation may therefore have a molecular origin. Owing to the amphiphilic nature of crown ethers, these molecules can form an intermediate layer between the polar enzyme surface and the apolar organic solvent, which facilitates the removal of water molecules from the active site upon substrate binding.[16] Crown ether molecules can act as carriers for water molecules in this process, since a relationship between the water-binding properties of several crown ethers and the enhancement of the enzyme activation has been observed.[9]

In conclusion, pretreatment of enzymes by lyophilisation in the presence of 18-C-6 results in enzymes with high activity under dry non-aqueous conditions. This enzyme activation procedure is a very easy method to overcome one of the most serious obstacles in the application of enzymes in organic solvents, *i.e.* low enzyme activity, and therefore significantly enlarges the possibilities to exploit the well-recognized advantages of non-aqueous enzymology.

We thank A. Hovestad and A. Montanaro-Christenhusz for technical assistance. This investigation was supported by the Netherlands Technology Foundation (STW), Technical Science Branch of the Netherlands Organization for Advanced and Pure Research (NWO) and DSM Research.

Received, 1st November 1994; Com. 4/06675H

Footnotes

† The reactions are first order in substrate and enzyme concentration; therefore, $V_o = k_{cat}/K_M \times [E] \times [S]$. The percentage of accessible active sites of α-chymotrypsin and subtilisin Carlsberg in cyclohexane–1 mol dm^{-3} propan-1-ol was estimated by a literature procedure[11] and was 13 ± 2% and 7 ± 2%, respectively.

‡ Although the crown ether activation of subtilisin Carlsberg is less pronounced than that of α-chymotrypsin it should be noted that the ultimate activity of the crown ether pretreated subtilisin Carlsberg is only 130 times lower than the activity of this enzyme in water (k_{cat}/K_M values 100 and 13000[11] $dm^3\ mol^{-1}\ s^{-1}$, respectively).

§ With *N*-acetyl-D,L-phenylalanine ethyl ester as substrate for both enzyme preparations, no D-propyl ester could be detected upon separation of the product on a chiral GC column. This experiment shows that pretreated α-chymotrypsin, like the unpretreated enzyme, is enantiospecific under these conditions and that a non-enzymatic transesterification reaction is not occurring.

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